Free bhCG (1st Trimester)
ELISA kit 96 Tests

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Enzyme immunoassay for the titration of free beta subunit of the human chorionic gonatropin (ß-hCG) in human serum

40-056-205074 (96 tests – 12 x 8 wells)

For Research Use only

1. INTENDED USE

ELIZEN Free β-hCG Kit is intended to the quantitative measurement of free β-subunit of the human chorionic gonadotropin in human serum.

2. INTRODUCTION

Human chorionic gonadotropin (hCG) is a glycoprotein with a molecular weight of 38000, normally produced by the placenta during
pregnancy. The hormone is present in blood and urine around seven to thirteen days following implantation of the fertilized ovum. Structurally intact hCG molecules consist of two non-covalently linked polypeptide subunits, the alpha and beta chain subunits. The α-subunit is common to all glycoprotein hormones and the β-subunit is responsible for the immunological and biological specificity.
Normal pregnancy is associated with an exponential increase of both holo-hCG and its free ß-subunit. ß-hCG appears in the sera of
pregnant women 5 days after the implantation of blastocyst and around the 10th week of pregnancy its concentration can reach up to 100 mIU/ml. This level gradually decreases during the 2nd and 3rd trimesters of pregnancy. After delivery, ß-hCG returns to undetectable level.

3. TEST PRINCIPLE

ELIZEN Free ß-hCG test is a sandwich ELISA: the wells of the microplate are coated with a monoclonal anti-ß-hCG. If ß-hCG is present in the sample, ß-hCG is captured by the antibodies immobilized on the microplate. After an incubation and a washing step
to remove unbound material, specific monoclonal antibodies anti-ßhCG conjugated to HRP are added and bind to ß-hCG.

So, the following complex is formed: **Mouse anti-ß-hCG IgG * ß-hCG *Mouse anti-ß-hCG peroxidase conjugate.**

After a second incubation followed by a washing step, the immunocomplex is detected by reaction with TMB substrate and the
development of a blue colour which changes into yellow by stopping the enzymatic reaction with sulfuric acid. The intensity of this colour is directly proportional to the amount of ßhCG in the sample.

Absorbance at 450 nm is read using an ELISA microtiter plate reader.

4. REAGENTS AND MATERIAL SUPPLIED
When stored at 2 – 8°C, all unopened reagents of th e kit will retain reactivity until expiry date. Do not use reagents beyond this date.

1. Microtiterplate: 96 breakable wells coated with anti-β-hCG. Keep unused wells at 2-8°C, protected from moisture in t he provided aluminium bag and carefully sealed. Immediately, after removal of strips, the remaining strips should be resealed in the outer bag along with the desiccant and stored at 2-8°C. It is important to ensure the desiccant remain s in the bag.

2. β-hCG **calibrators:** 7 vials calibrators supplied in human serum. Preservative : thymol . Store at 2 – 8°C. Reconstitute the Zero calibrator with 3.5 ml and other calibrators with 0.8 ml of fresh and germ free distilled water, mix well. The reconstituted calibrators are stable one week at 2-8 °C. For longer storage periods, aliquots should be kept at –20°C. Avoid successive freeze thaws cycles.

The calibrators are calibrated against the IRP 75/551 from NIBSC (U.K)-1 ng= 1 mIU
Check on the box of each lot for the accurate values of the calibrators and controls.

3. Enzyme conjugate: 1 vial (0.25 ml) of monoclonal antibody anti-β-hCG conjugated to horseradish peroxidase (HRPO). Preservative: thimerosal 0.02 %. Dilute 100x concentrated enzyme conjugate with the **dilution buffer**. The diluted enzyme conjugate is stable for 24 h at 2 – 8 °C.

Store concentrated at 2 – 8°C.

4. Control serum 1 & 2: one set of 2 vials, each containing a lyophilized human serum (preservatives :gentamycin and proclin)
with two different amounts of ß-hCG (low and medium concentration). Reconstitute each vial with 0,8 ml of H2O, mix well.
After reconstitution the controls are stable one week at 2-8 °C. For longer storage periods, aliquots should be kept at –20°C. Avoid
successive freeze thaws cycles.
For the expected value refer to the sheet in the box.
Store lyophilized at 2-8 °C.

5. Washing solution: 1 vial (100 ml) of 15 x concentrated buffer with Tween 20. Preservative: thimerosal 0.01 %. Bring the vial content to 100 + 1400 = 1500 ml (final volume) with fresh and germ free distilled water. The diluted washing solution is stable for 1 week at 2-8°C.
Store concentrated at 2 – 8°C

6. Chromogen substrate: 1 vial (13ml) of 3,3' , 5,5' Tetra-methylbenzidine.
Ready for use.
Store at 2 – 8°C

7. Blocking reagent: 1 vial (15ml) of 0.5M H2SO4. Ready for use.Irritant agent.
Stable at 2 – 8°C

8. Dilution buffer: One vial (35 ml) of phosphate buffer with BSA and preservative (Thimerosal). Ready to use. Store refrigerated. Stable at 2-8°C.
- Sealing tape
- Strip holder
- Instruction leaflet

KIT REAGENTS

Reagents	Quantity	Physical state
Microtiterplate	96	Ready for use
Calibrators 0	2 x 3.5 ml	Lyophilised
Calibrators 1 – 5	5 x 0.8 ml	Lyophilised
Control Sera	2 x 0.8 ml	Lyophilised
Enzyme conjugate	1 x 0.25 ml	Concentrated 100 x
Washing Solution	1 x 100 ml	Concentrated 15 x
Chromogen Substrate	1 x 13 ml	Ready for use
Blocking Reagent	1 x 15 ml	Ready for use
Dilution buffer	1 x 35 ml	Ready for use

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- ELISA microplate reader, equipped for the measurement of absorbance at 450/620nm
- Incubator 37°C
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex tube mixer
- Deionised or (freshly) distilled water
- Disposable tubes
- Timer

6. PRECAUTIONS

A. Safety

1. This kit is for in vitro diagnostic only.
2. Washing solution, peroxidase conjugate and calibrators contain Thimerosal. This product is highly toxic by inhalation, swallowing
and contact with skin. Keep away from food and drink.
3. Wear protective clothes and gloves when handling the samples. Microbial contamination of reagents or specimens may give false
results. Samples should be incinerated after being tested. In case of contact with skin or eyes, rinse thoroughly with water. In case of accident, consult immediately a physician and show him/her the product label.
4. Do not smoke, eat, drink or apply cosmetics in areas where kits and blood specimens are handled.
5. The reagents have been tested for HBs Ag and anti-HIV antibodies and have been found to be non-reactive!, However, all material should still be regarded and handled as potentially infectious.

B. Operating

1. Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
2. Do not freeze kits.
3. Do not use kit components after the expiration dates stated on their labels.
4. Do not keep diluted reagents for longer that the recommended periods.
5. Keep all reagents at normal refrigerator temperature (2-8°C) in closed containers when not in use.
6. Ensure that all reagents are equilibrated to 18-25°C before use.
7. Do not use any solutions which have become turbid.
8. The strips, calibrators and internal controls are vacuum sealed and packed in an outer aluminium pouch containing a desiccant.
Immediately after removal of strips, the remaining strips should be resealed or closed with a Scotch tape in the outer bag along with
the desiccant and stored at 2-8°C. It is important to ensure the desiccant remains in the bag.
9. The general purpose reagents washing solution and stopping solution are interchangeable between different lots while all other
reagents are specific for the individual package lot and must not be interchanged with other lots.
10. Do not use reagents from other manufacturers along with the kit reagents for a given test run.
11. Do not interchange reagent vials and their screw caps to avoid cross-contamination. Use a clean, fresh, disposable pipette tip for each reagent or specimen manipulation.
12. Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.

7. SPECIMEN COLLECTION

Use serum samples.
The clotted samples must be centrifuged as soon as possible to avoid subunit dissociation. No special pretreatment of the sample is
necessary. Do not use hemolyzed or lipemic specimens.
Storage: serum samples can be kept at 2-8°C for maximum 24 h. Avoid multiple freeze-thaw cycles for any specimen.
If a specimen is expected or known to have a concentration above the highest calibrator it has to be diluted with the zero calibrator to fall within the measuring interval.

8. TEST PROCEDURE

General remarks.

- Results reliability depends on strict adherence to the described procedure.
- Bring all reagents, samples and calibrators to room temperature before use.
- All reagents must be mixed without foaming.
- Prior to starting the assay, the distribution and identification plan for all specimens and calibrators should be carefully established.
- Select required number of microtiter strips and place in the strip holder.
- Allow 1 well for the substrate blank and 6 wells for the calibratorsbut it is recommended to perform the standard curve in duplicates. A standard curve must be included in each assay.

Step 1
1. Leave well A1 for substrate blank.
Pipette reconstituted calibrators, controls and samples in the following order:
Dispense directly in the well 10 µl of serum + 200 µl of **dilution buffer** and shake gently.
2. Cover wells with foil or adhesive film.
3. **Incubate 60min at 37°C** .
4. Aspirate off contents of wells and add to each well 4 times 300 µl of **working washing solution** and aspirate off again.

If an automatic washer is used, primarily wash with **working washing solution** and repeat 4 times. Ensure the washer fills all wells completely and aspirates off efficiently between washing steps (remaining liquid < 15 µl) !

At the end carefully remove remaining fluid by tapping the strips on tissue paper prior to next step.

Step 2
1. Dispense 100 µl of prediluted **anti-ß-hCG peroxidase conjugate** (see reagents and material supplied) in all wells except well A1.
2. Cover wells with foil or adhesive film.
3. **Incubate 60min at 37°C** .
4. Aspirate off contents of wells and add to each well 4 times 300 µl of **working washing solution** and aspirate off again.

Step 3
1. Dispense 100 µl of **TMB solution** to all wells.
2. **Incubate 10 min at room temperature** (15-30°C) in the dark
3. Stop the enzymatic reaction by addition of 100 µl of **Blocking reagent** to all wells.
4. Zero the ELISA microtiter plate reader using the substrate blank in well A1.
5. Measure the absorbance at 450 nm (A450).

Note: Dual wavelength reading using 620 nm or 690 nm as reference wavelength is recommended but not compulsory.
Strips should be read within 15 min.

9. INTERPRETATION OF RESULTS

A. Titration curves

Automatically: select semi-log Cubic Spline graph on the microplate reader.
Manually: for each parameter, draw a graph as follows:

1. ORDINATES: measured optical density value for each Calibrator
2. ABSCISSAE: decimal logarithm of concentrations of each Calibrator

CALIBRATORS APPROXIMATE VALUES :

	ß-hCG (mIU/ml)	OD
Cal 0	0	0.010
Cal 1	2	0.104
Cal 2	8	0.257
Cal 3	35	1.002
Cal 4	90	2.175
Cal 5	130	2.483

	Mean (mIU/ml)	Expected Range
Control 1	26.3	(20.11-30.16)
Control 2	61.1	(53.53-80.29)

Plot optical density values of the samples on the curve
O.D. = f (Log concentration C)
Read in abscissae concentration logarithm (X)
Calculate C (concentration): **C = 10 X**

CAUTION
The samples showing a concentration exceeding that of the calibrator C5 should be prediluted in **the zero calibrator** and tested again. The concentration thus obtained has to be multiplied by the dilution factor.

B. Validation of the test
The test may be considered valid provided the following criteria are met:
1. The substrate blank in well A1 appears colourless to the eye
2. C0 ≤ 0.1
3. C5 ≥ 1.5

10. PERFORMANCES OF THE ASSAY

SPECIFICITY: The present method has shown the following crossreaction. The specificity was estimated by spiking a pool of serum where free ß-hCG concentration was lower than 0.01 ng/ml with the following preparations (Quantity added from 0 to 10µg/ml):

Compound added	No Cross-reaction observed until:
hLH	< 5µg/ml
hFSH	< 1µg/ml
hTSH	< 4µg/ml
ßLH	< 0.5µg/ml
αhCG	No cross reaction

SENSITIVITY: The sensitivity was calculated based upon the standard curve and expressed as the minimal dose showing a significant difference from the Zero Standard (mean value + 3 S.D.) This dose is 0.87 mIU/ml.

PRECISION: Precision was evaluated upon intra- and inter- assay variability, in 3 sera at different free ß-hCG concentrations.

Intra-Assay

Serum	Mean ± SD mIU/ml	CV %	Replicates
A	22.6 ± 2.0	8.9	20
B	44.5 ± 1.3	2.8	20
C	71.7 ± 3.1	4.4	20

Inter-Assay

Serum	Mean ± SD mIU/ml	CV %	Replicates
A	24.9 ± 2.2	8.7	9
B	49.6 ± 3.0	6.1	9
C	61.1 ± 2.9	4.7	9

ACCURACY: Accuracy of the method has been checked as follows :
Recovery test : known amounts of free ß-hCG have been added to a pool of normal sera and tested.

Endogenous (mIU/ml)	Added (mIU/ml)	Expected (mIU/ml)	Measured (mIU/ml))	Recovery (%)
11.5	1.0	12.5	11.9	95.3
	4.0	15.5	14.7	95.2
	17.5	29	28.2	97.3
	45.0	56.5	60.4	107.0

Parallelism Test: One serum with high concentration was tested at different dilutions with the Zero standard.

Dilution	Expected (mIU/ml)	Measured (mIU/ml)	Ratio %
	29.6		
1/2	14.8	17.0	115
1/4	7.4	8.2	111
1/8	3.7	2.9	78

11. METHOD COMPARISON

The performance of ELIZEN Free β-hCG ELISA has been assessed by determination of free beta HCG concentration in 150 samples in comparison with another commercially available kit. The correlation was > 90%.

12. LIMITATIONS OF USE

Heterophilic antibodies in human serum can react with the immunoglobulins included in the assay components causing interference with *in vitro* immunoassays. Samples from patients routinely exposed to animals or animal serum products can demonstrate this type of interference potentially causing an anormalous result. The reagents of the kit have been formulated to minimize the risk of interference; however, potential interactions between rare sera and test components can occur.

13. BIBLIOGRAPHY

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